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TITLE:
Identification, Characterization, and Utilization of Adult Meniscal Progenitor Cells

PRINCIPAL INVESTIGATOR: Dr. Vicki Rosen

CONTRACTING ORGANIZATION: Harvard University, Boston
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14. ABSTRACT

Meniscal injuries are the most common traumatic leg injuries, accounting for over half of the knee arthroscopies performed each year. Damaged menisci rarely regain normal structural integrity or mechanical strength, and surgical repair cannot reliably prevent the degenerative changes that occur post injury and presage the development of knee osteoarthritis (OA). New treatments centered on the stem/progenitor cell population resident within the adult meniscus will be key to derailing the connection between acute meniscal injury and post-traumatic knee OA. Here we combine mouse genetics with molecular and cell biology to develop a profile of repair cells in the adult meniscus, track meniscal stem/progenitor cell (MSPC) behavior within meniscus as function of age, and assess the contribution of resident MSPCs to repair after meniscal injury. During the current research period we made significant progress toward our goals by establishing a standard protocol for harvesting MSPCs from 8 week, 6 month and 1-year old mouse menisci. MSPCs grow as colonies, express stem cell and meniscal gene signature markers found in adult human meniscus, and can be successfully passaged. We also piloted a novel mouse meniscal tear injury model, and are now ready to use this technique for experiments outlined in our proposal.

15. SUBJECT TERMS
meniscus, meniscal cells, stem cells, progenitor cells, meniscus healing, meniscus repair, osteoarthritis

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1. Introduction

Meniscal injuries are the most common of traumatic knee injuries. Once damaged, meniscal tissue rarely regains normal structural integrity and mechanical strength. Surgical repair of meniscal tears cannot reliably prevent the degenerative changes that occur after injury. As a result, meniscal injuries are a common underlying cause of post-traumatic osteoarthritis. This is particularly striking in young, healthy individuals such as military personnel, where meniscal injury is often associated with long-term disability and knee replacement surgery. This proposal focuses on identifying meniscal stem/progenitor cells (MSPC) in the adult meniscus, developing a molecular profile of these cells, and examining the contribution MSPC provide to repair after meniscal injury. Information gathered from these studies will be useful for developing new treatments for acute meniscal injuries, lessening the need for joint replacement and reducing long-term disability in active adults.

2. Keywords

meniscus, meniscal cells, stem cells, progenitor cells, meniscus healing, meniscus repair, osteoarthritis

3. Accomplishments

Major goals of project:

1. Isolation and characterization of primary meniscus cells (MSPC) from genetically labeled mice.
2. Interaction of MSPC with meniscus niche in vivo.
3. Functional assessment of healing meniscal tears by MSPC in vivo.

Accomplishments in this review period:

1. Isolation and characterization of MSPC.

Task 1: Obtain mice necessary for experiments. This task has been completed.

Task 2: Develop standard MSPC isolation protocol. This task has been completed and previously reported.

Task 3: Characterize MSPC.

Subtask (3a): Stem cell-like cell characteristics of MSPCs will be assessed using flow cytometry. P1 MSPCs from 8 wk old mice were suspended at a density of 1 x10^6 cells in FACS buffer (PBS, 1% FBS) containing 1-5 mg/ml of primary or control antibodies. After incubation for 30 minutes at 4^{o}C, the cells were washed 3 times with FACS buffer and suspended in 500 ml FACS buffer for the analysis. The following Fluorescein isothiocyanate (FITC), allophycocyanin (APC) or phycoerythrin (PE) coupled antibodies were used: CD34, CD44, CD73, CD105, Sca1 and CD90.2. Samples were evaluated using a BDLSRII flow cytometer and the data analyzed using the Flowjo software program. To confirm that MSPCs possess the established properties of stem cells, they were subjected to fluorescence-activated cell sorting (FACS) analysis for surface markers associated with stem cells (Figure 1). We found that MSPCs (P1) were over 97% positive for CD44, a mesenchymal stromal cell marker and over 87% positive for stem cell antigen-1 (Sca-1). In addition, cells were also positive for CD90.2 (54%) and CD73 (35.4%) and expressed low levels of CD105 (2.2%). MSPCs were negative for CD34 verifying the lack of contaminating hematopoietic cells.

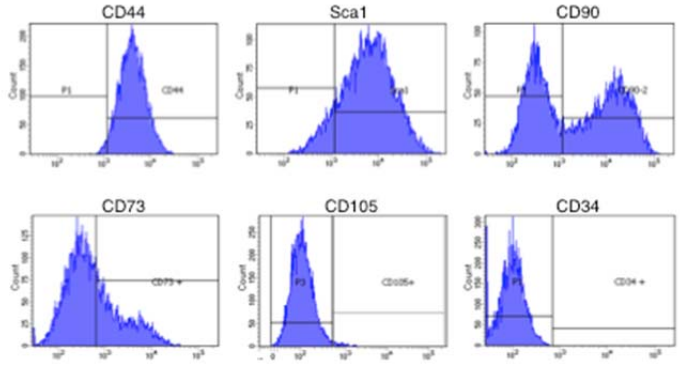

Figure 1. Characterization of MSPCs. (A) Flow cytometry analysis of the expression of cell surface markers related to stem cells. MSPCs were 97.3% positive for CD44, 87.8% positive for Sca-1, 54% positive for CD90, 35.4% positive for CD73, 2.2% positive for CD105 and 0.1% positive for CD34.

Subtask (3b): Clonogenicity of the population will be determined by examining the ability of single cells to form colonies. This task has been completed and previously reported.

Subtask (3c): RNA collected from each colony will be analyzed for expression of meniscus gene signature and for stemness markers. This task has been completed and previously reported.

Subtask (3d): Multipotentiality of the MSPCs will be evaluated.

For multi-differentiation assays, MSPCs from 8wk old mice were plated at colony forming density in 25cm^2 flasks and cultured in growth medium for 12 days. To analyze osteogenic potential, cells were switched to growth media supplemented with 10 mM β-glycerol phosphate, 50 mg/ml ascorbic acid for another 7 days. Colonies were then stained with 0.5% Alizarin Red S. To test adipogenic potential, cells were switched to growth media supplemented with 100 nM dexamethasone, 5 mg/ml insulin, 50 mM indomethacin for 7 more days. Colonies were then stained with 0.3% Oil Red O. To assess chondrogenic potential, cells were switched to growth media supplemented with 50 mg/ml ascorbic acid, 100 nM dexamethasone 40 mg/ml L-proline, 2mM sodium pyruvate and 1% insulin transferrin-selenious acid mix (ITS+) for another 7 days. Flasks were then stained with Alcian Blue pH 1.0. We examined whether MSPCs had the capacity to differentiate into various cell lineages (osteoblast, adipocyte, chondrocyte), a fundamental characteristic of stem cells. To test the osteogenic potential of this cell population, MSPC colonies were differentiated in osteogenic medium. After 7 days, Alizarin Red positive mineralized calcium deposits were detected (Figure 2). After induction in adipogenic medium for 7 days, MSPC colonies showed accumulation of lipid droplets visualized by positive oil red O staining (Figure 2). When differentiated in chondrogenic medium for 7 days, MSPC colonies stained positive for Alcian Blue indicating synthesis of proteoglycans by chondrocytes (Figure 2). These results suggest that MSPCs are multipotential, a key feature of mesenchymal stem cells.

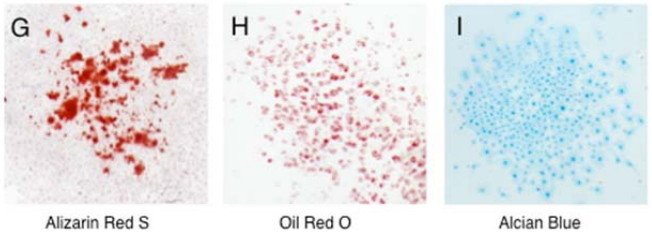

Figure 2. Analysis of stem cell features of MSPCs. The multidifferentiation potential of MSPCs. (G) Alizarin Red S staining shows osteogenic differentiation of MSPCs. (H) Oil Red O staining shows adipogenic differentiation of MSPCs. (I) Alcian Blue staining shows chondrogenic differentiation of MSPCs.

Subtask (3e): Self-renewal capacity of MSPCs will be examined by calculating proliferation rates using a BrdU assay (Invitrogen), and doubling times will be determined. This task is currently being completed.

Subtask (3f): Migratory capacity of MSPCs will be determined and test substances IGF1, BMP2, and TGFβ evaluated. A reproducible protocol for this task is currently being tested.

2. Interaction of MSPC with meniscus niche in vivo.

Task 1: Obtain and age mice for experiments. This task has been completed.

Task 2: Introduce MSPC into meniscus in vivo.

We have experienced difficulty in injecting meniscal cells into the mouse meniscus. The small size and accessibility of the mouse knee has been a challenge. Due to these issues, we are developing an in vitro culture system for mouse meniscus where we can make an injury and the inject MSPCs. To begin this process, we isolated menisci from 8 wk old mice and placed them in 6 well plates with various media and amounts of FBS to determine the optimal culture conditions. The meniscal tissue was viable for at least 5 days when cultured in 1 ml of DMEM with or without FBS (Figure 3). Next, we tried making a small injury in the meniscus using a 30 gauge syringe needle. We were able to reproducibly make a radial or oblique tear in the cultured meniscus (Figure 3). We plan to continue developing this in vitro model and inject MSPCs into the intact and injured meniscus. We will use the knowledge and practice this in vitro model affords us to

continue to develop a protocol for injecting MSPCs making an injury in the meniscus in vivo.

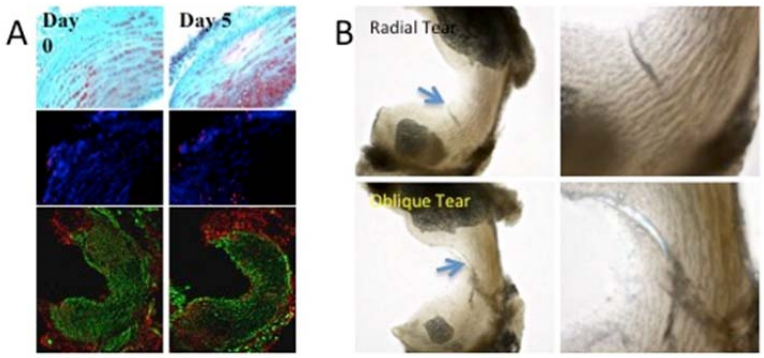

Figure 3. In vitro culture system for mouse meniscus. (A) Meniscus from 8 wk old mouse cultured for 5 days are viable as shown by Safranin O and Fast Green staining, TUNEL staining and Live/Dead Cytotoxicity staining where green fluorescent cells are alive. (B) Visible radial and oblique tears can be made in 8wk mouse meniscus cultured in vitro.

Task 3: Track MSPC behavior. This has not yet been attempted due to difficulties with Task 2.

Task 4: Analyze MSPC niche in adult meniscus. Preliminary data have been collected in young mice and task will be completed on aged mice during this period. To verify the data obtained from our FACs and qPCR analyses with MSPC behavior *in vivo*, we examined the spatial localization of a select group of MSPC expressed factors using immunohistochemistry. CD44, biglycan, Lox and IGF-1 were all detected in the outer periphery of the meniscus in the fibro-chondrocytes of the superficial zone in knees of 8 wk old mice (Figure 4A-F). This superficial zone is thought to contain endogenous progenitor cells with regenerative capabilities. In addition, positive staining was seen for CD44, biglycan, Lox and IGF-1 in the fibroblast like cells of the outer vascular zone. This region is rich in collagen type I and has a higher capacity for healing and repair. These data confirm our *in vitro* studies and suggest that in the mouse, endogenous progenitor cells may reside in the superficial and outer region of the meniscus *in vivo*.

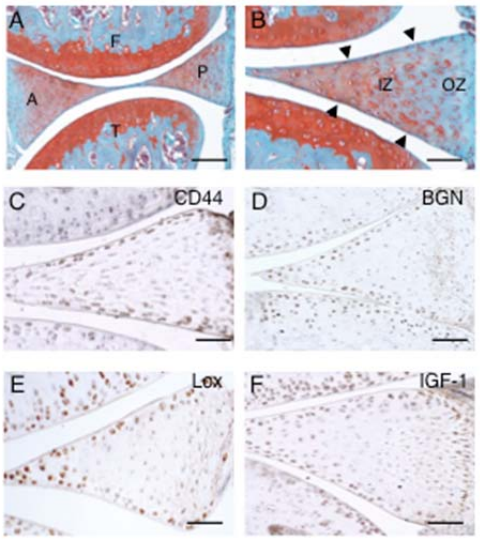

Figure 4. Localization of proteins associated with MSPCs in mouse knee. (A) Safranin O staining of 8 wk old mouse knee. Scale bar = 200µm. F, femur; T, tibia; A, anterior meniscal horn; P, posterior meniscal horn. (B) High power view of posterior meniscal horn. Scale bar = 100µm . Arrowheads point to the superficial zone. IZ, inner zone of meniscus; OZ, outer zone of meniscus. Immunohistochemistry for (C) CD44 (D) Biglycan (BGN) (E) Lysyl oxidase (Lox) and (F) IGF-1 proteins showed positive staining of cells in the superficial and outer zone of posterior horn of the 8 wk meniscus. Scale bar = 100µm.

Task 5: Assess functional changes in meniscus due to addition of MSPC: These studies have not been started but will begin after the completion of Task 2. We also plan on analyzing functional changes in the meniscus due to addition of MSPC using tissue from our in vitro culture system.

Task 6: Assess effects of niche age on MSPC: These studies have not begun but will begin after the completion of Task 2.

3. Functional assessment of healing by MSPC.

Task 1: Obtain mice for experiments: Mice will not be available until December 2016 and all subsequent tasks require these mice, so these studies will not begin until then.

Task 2: Introduction of MSPC into adult meniscus. This task has not yet been attempted due to availability of mice of proper ages and the difficulties outlined above with injection of MSPCs in to mouse meniscus in vivo.

Task 3: Production of meniscus tear. This task has not yet been attempted due to availability of mice of proper ages and the difficulties outlined above with injection of MSPCs in to mouse meniscus in vivo.

Task 4: Tracking MSPC participation in repair. This task has not yet been attempted due to availability of mice of proper ages and the difficulties outlined above with injection of MSPCs in to mouse meniscus in vivo. However, the conditions for immunohistochemistry and histological analysis have been worked out – see Figure 4 and Task 4 under Specific Aim 2.

Task 5: Analysis of repair process. This task has not yet been attempted due to availability of mice of proper ages and the difficulties outlined above with injection of MSPCs in to mouse meniscus in vivo.

Task 6: Effect of enhancing signaling on repair. This task has not yet been attempted due to availability of mice of proper ages and the difficulties outlined above with injection of MSPCs in to mouse meniscus in vivo.

Task 7: Effect of adding exogenous MSPC on repair. This task has not yet been attempted due to availability of mice of proper ages and the difficulties outlined above with injection of MSPCs in to mouse meniscus in vivo.

Training and professional development opportunities provided by the project:
The project provided training opportunities for a postdoctoral fellow (Rui Rui Shi) to learn many new laboratory skills including explant culture and FACs analysis and an undergraduate summer student (Dylan Mathewson) to learn about the biology of the meniscus as well as techniques such as histology, immunohistochemistry and cell culture. This project has also allowed Vicki Rosen and Laura Gamer to participate in the newly formed Meniscus Section at the Orthopaedic Research Society meeting in 2016 and this resulted in increased knowledge of meniscal biology and repair. In addition, our

collaboration with Lin Han at Drexel University has allowed us to gain expertise in measuring tissue strength in relation to structure.

How were results disseminated to communities of interest?

The results of our studies thus far have been submitted in August of 2016 for publication in the journal Connective Tissue Research and for presentation at the 2017 Orthopaedic Research Society meeting.

What do you plan to do in the next reporting period to accomplish the goals?

Nothing to report.

4. Impact

Impact on principal discipline:

A detailed understanding of the molecular and cellular events that underlie tissue repair is essential for addressing pathologies associated with injury, disease and aging in the musculoskeletal system. Little information exists about resident stem cell populations in the mouse meniscus, a model increasingly used to study joint diseases including OA. In this report, we detail the isolation and characterization of resident meniscus stem/progenitor cells (MSPC) derived from adult mouse meniscus explant cultures. MSPCs demonstrate general properties of other stem cells including clonogenicity and multi-potentiality, express cell surface antigens associated with mesenchymal stem cells, and display elevated levels of meniscal marker genes. In addition, we observe a correlation between sites within the meniscus that are thought to harbor endogenous repair cells and the localization of proteins associated with MSPCs. We believe that identification of MSPCs provides a powerful tool for enhancing cell-based strategies focused on meniscal regeneration as there is increasing evidence that tissue-resident stem cells are critical for organ homeostasis and effective wound healing. Although MSCs and synovium-derived stem cells have been tested in meniscal injury models, endogenous stem/progenitor cells may be best suited for repair of the meniscus, a tissue with distinct composition, architecture and function in the knee joint.

In previous studies, meniscus-derived stem cells were identified and isolated from rabbit and human following tissue digestion. Unlike those investigations, the MSPCs described in our studies migrated out of whole explanted mouse menisci. Since cell motility is now recognized as a vital property of stem and progenitor cells, our MSPCs may represent a more promising cell population for meniscal repair. The MSPCs we identified express progenitor and stem cell markers that localize to the outer vascular zone and superficial zone of the adult meniscus, two areas thought to contain cells for repair. As these anatomical sites are distinct in character, the presence of MSPCs at both locations suggests the possibility of multiple stem cell niches within the meniscus.

Our current findings support the notion that a greater understanding of the basic biology of meniscal stem/progenitor cells is needed to better inform cell based treatments for meniscal pathologies. Analysis of meniscus derived-stem cells in an animal model such as the mouse will allow for detailed studies of their behavior during injury and repair and of the regulatory pathways that guide these processes, critical steps in identifying therapeutic targets for the regeneration of diseased or injured meniscal tissue.

Impact on other disciplines: Nothing to report.

Impact on technology transfer: Nothing to report.

Impact on society: Nothing to report.

5. Changes/Problems

Changes in approach: Due to the small size and accessibility of the mouse knee, we have experienced difficulty in injecting meniscal cells and making a reproducible injury in the mouse meniscus. To aid in working out the problems with the in vivo model, we are developing an in vitro culture system for mouse meniscus where we can make an injury and the inject MSPCs. The lessons learned from the in vitro model will be applied to the mouse surgeries and injections.

Actual or anticipated problems or delays: We have had some delay in the progress of Aims 2 and 3 due to difficulties in getting the in vivo surgical technique and injection protocols worked out and waiting to age mice out to get the appropriate time points (52 wk).

Changes with impact on expenditures: Our progress with this project has been slowed by the previously mentioned problems as well as a postdoctoral fellow resigning from the lab due to personal and family issues. These were the reason for our request for a No Cost Extension.

Changes in use or care of animals/biohazards/select agents. Nothing to report.

6. Products

Journal publications:

Identification and Characterization of Adult Mouse Meniscus Stem/Progenitor Cells. Laura W. Gamer, Rui Rui Shi, Ashira Gendelman, Dylan Mathewson, Jackson Gamer and Vicki Rosen. Manuscript submitted on August 25, 2016 to Connective Tissue Research for the Special Issue on Meniscus to be published in March of 2017.

Other publications, conference papers and presentations:

Isolation and Characterization of Adult Mouse Meniscus Stem/Progenitor Cells. Laura Gamer, Rui Rui Shi, Ashira Gendelman, Dylan Mathewson, Jackson Gamer and Vicki Rosen. Abstract submitted for Orthopaedic Research Society meeting in 2017.

Technologies or techniques: MSPC isolation protocol will be shared in Connective Tissue Research journal article.

Inventions, patent applications and/or licenses: Nothing to report.

Other products: Nothing to report.

7. Participants and other collaborating organizations.

Individuals who have worked on the project:

Vicki Rosen, Professor. No Change.

Laura Gamer, Instructor. No Change.

RuiRui Shi, Post-doctoral Research Fellow. No Change.

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

There has been a change in the active other support of the PI, Dr. Vicki Rosen, since the last reporting period. The following project has ended:

Apr 1, 2013 - Mar 31, 2016
NIH/NIAMS R21AR063955-02
"BMP2 signaling of the development of osteoarthritis"

What other organizations were involved as partners?

Nothing to Report.

8. Special reporting requirements:

Nothing to report.

9. Appendices

Isolation and Characterization of Adult Mouse Meniscus Stem/Progenitor Cells.
Laura Gamer, Rui Rui Shi, Ashira Gendelman, Dylan Mathewson, Jackson Gamer and Vicki Rosen. Abstract submitted for Orthopaedic Research Society meeting in 2017.

Identification and Characterization of Adult Mouse Meniscus Stem/Progenitor Cells.
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Identification and Characterization of Adult Mouse Meniscus Stem/Progenitor Cells

Laura Gamer[1], Rui Rui Shi[1], Ashira Gendelman[1], Dylan Mathewson[1], Jackson Gamer[1] and Vicki Rosen[1]
Harvard School of Dental Medicine, Boston, MA[1]

Disclosures: Laura Gamer (N), Rui Rui Shi (N), Ashira Gendelman (N), Dylan Mathewson (N), Jackson Gamer (N),Vicki Rosen (N).

INTRODUCTION: The most common knee injury is damage to the meniscus, a fibrocartilaginous cushion with a pivotal role in protecting the articular cartilage from damage during movement. Although treatment of acute meniscal injuries has evolved dramatically in recent years, surgical procedures aimed at repairing or replacing damaged menisci are often unsuccessful. In fact, most surgical repair of meniscal tears cannot reliably prevent the progression of degenerative changes and clinical symptoms that presage the development of knee osteoarthritis (OA). Attempts to enhance meniscal healing with addition of fibrin clots or growth factors have shown some promise, consistent with the idea that the intrinsic healing potential of the meniscus might be improved by activation of endogenous meniscal stem cells. However, progress in this area has been limited by a lack of information about the origin of meniscal progenitors and the signaling pathways controlling their proliferation and differentiation. A greater understanding of the basic biology of meniscus-derived stem cells will be necessary for their application in cell based repair and tissue engineering strategies. To this end, we isolated and characterized meniscal stem progenitor cells (MSPCs) from adult mouse meniscus. We chose to analyze murine cells because our data could be used in future studies on the regulatory mechanisms underlying meniscal regeneration and the mouse is an ideal system for genetic manipulation. Mouse MSPCs exhibit the general features of tissue-specific stem cells isolated from other musculoskeletal tissues, including clonogenicity, multi-potency and expression of several common cell surface markers. In addition, adult mouse MSPCs express significant levels of genes first identified in embryonic mouse meniscus that may be important for meniscal formation. We also show that markers associated with MSPCs localize in distinct regions of the adult mouse meniscus hypothesized to harbor cells capable of responding to meniscal injury.

METHODS: This study was approved by the Harvard Medical School IACUC. MSPCs were isolated from C57Bl/6 mouse menisci grown in explant culture. These cells were characterized for stem cell properties using colony-forming assays and for their ability to differentiate in osteogenic, adipogenic and chondrogenic media. Flow cytometry was used to detect the presence of surface antigens related to stem cells on MSPC, and qRT-PCR was used to examine the gene expression profile of MSPCs. The major proteins associated with MSPCs were localized in the adult mouse knee using immunohistochemistry (IHC).

RESULTS: Based on explant culture procedures for human meniscus and cartilage, a protocol was developed for isolating meniscal progenitor cells from adult mouse meniscus. After 5-7 days, cells began to grow out of the explanted menisci (Figure 1A). In culture, these meniscus-derived cells grew clonally and exhibited a spindle-shaped morphology (Figure 1B). Cells grew out of both the lateral and medial menisci of mice of all ages tested (8wk, 6 mo, 1yr) and grew well in monolayer. Mouse MSPCs showed universal stem cell like characteristics including clonogenicity and multi-potentiality (Figure 1C-F). FACs analysis revealed MSPCs expressed the mesenchymal stem cell markers CD44, Sca-1, CD90 and CD73, and when cultured in monolayer had elevated levels of biglycan and collagen type I, important extracellular matrix components of adult meniscus. MSPCs also expressed robust levels of the meniscus signature gene *lysyl oxidase* (*Lox*), an enzyme responsible for collagen cross-links in skeletal and connective tissue, as well as *Igf-1*, the major signaling pathway enriched in the developing meniscus. To verify data obtained from FACs and qPCR analyses with MSPC behavior *in vivo*, the spatial localization of a select group of MSPC expressed factors was examined using IHC. CD44, biglycan, Lox and IGF-1 were all detected in the outer periphery of the meniscus in the fibro-chondrocytes of the superficial zone of 8 wk old mice (Figure 2B-E). This superficial zone is thought to contain endogenous progenitor cells with regenerative capabilities. In addition, positive staining was seen for CD44, biglycan, Lox and IGF-1 in the fibroblast like cells of the outer vascular zone. This region is rich in collagen type I and has a higher capacity for healing and repair.

DISCUSSION: We believe that identification of MSPCs provides a powerful tool for enhancing cell-based strategies focused on meniscal regeneration, as there is increasing evidence that tissue-resident stem cells are critical for organ homeostasis and effective wound healing. Although MSCs and synovium-derived stem cells have been tested in meniscal injury models, endogenous stem/progenitor cells may be best suited for repair of the meniscus, a tissue with distinct composition, architecture and function in the knee joint.

SIGNIFICANCE: A greater understanding of the basic biology of meniscal stem/progenitor cells is needed to enhance treatments for meniscal pathologies. Analysis of meniscus derived-stem cells in animal models such as the mouse will allow for detailed studies of their behavior during injury and repair and of the regulatory pathways that guide these processes, critical steps in identifying therapeutic targets for the regeneration of diseased or injured meniscal tissue.

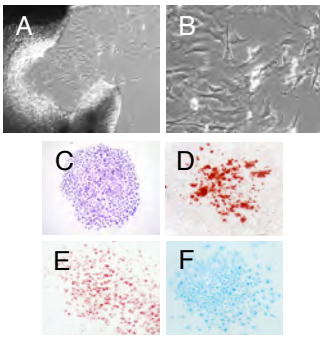

Figure 1. Isolation and analysis of MSPCs. (A) Cells growing out of lateral meniscus after 5 days in culture. (B) Spindle shaped morphology of these cells. (C) Sample colony formed by MSPCs stained with methyl violet. Multi-differentiation potential of MSPCs. (D) Alizarin Red S staining showing osteogenic differentiation. (E) Oil Red O staining showing adipogenic differentiation. (F) Alcian Blue staining showing chondrogenic differentiation.

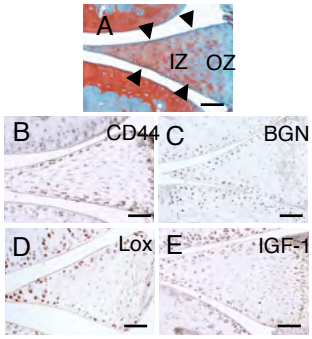

Figure 2. Localization of proteins associated with MSPCs in 8 wk mouse knee. (A) Safranin O of posterior meniscal horn. Arrowheads, superficial zone; IZ, inner zone; OZ, outer zone. IHC for (C) CD44 (D) Biglycan (E) Lox (F) IGF-1. Positive staining is detected in the superficial and outer zone of posterior horn of the meniscus. Scale bar = 100μm.

Identification and Characterization of Adult Mouse Meniscus Stem/Progenitor Cells

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Keywords:	meniscus, mouse, stem cell, osteoarthritis, progenitor cell

Identification and Characterization of Adult Mouse Meniscus Stem/Progenitor Cells

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Abstract:

Meniscal damage is a common problem that accelerates the onset of knee osteoarthritis. Stem cell based tissue engineering treatment approaches have shown promise in preserving meniscal tissue and restoring meniscal function. The purpose of our study was to identify meniscus-derived stem/progenitor cells (MSPCs) from mouse, a model system that allows for *in vivo* analysis of the mechanisms underlying meniscal injury and healing. MSPCs were isolated from murine menisci grown in explant culture and characterized for stem cell properties. Flow cytometry was used to detect the presence of surface antigens related to stem cells, and qRT-PCR was used to examine the gene expression profile of MSPCs. Major proteins associated with MSPCs were localized in the adult mouse knee using immunohistochemistry. Our data show that MSPCs have universal stem cell like properties including clonogenicity and multi-potentiality. MSPCs expressed the mesenchymal stem cell markers CD44, Sca-1, CD90 and CD73, and when cultured had elevated levels of biglycan and collagen type I, important extracellular matrix components of adult meniscus. MSPC also expressed significant levels of *Lox* and *Igf-1*, genes associated with the embryonic meniscus. Localization studies showed staining for these same proteins in the superficial and outer zones of the adult mouse meniscus, regions thought to harbor endogenous repair cells. MSPCs represent a novel resident stem cell population in the murine meniscus. Analysis of MSPCs in mice will allow for a greater understanding of the cell biology of the meniscus, essential information for enhancing therapeutic strategies for treating knee joint injury and disease.

Keywords: mouse meniscus, progenitor cells, stem cells, osteoarthritis

Introduction

The most common knee injury is damage to the meniscus, a fibrocartilaginous cushion with a pivotal role in protecting the articular cartilages of the tibia and femur from damage during movement. Although treatment of acute meniscal injuries has evolved dramatically in response to an increased understanding of the roles performed by the meniscus within the knee joint, surgical procedures aimed at repairing or replacing damaged menisci are often unsuccessful (1,2). In fact, recent data indicate that surgical repair of meniscal tears cannot reliably prevent the progression of degenerative changes and clinical symptoms that occur post meniscal injury and presage the development of knee osteoarthritis (OA) (3). Attempts to enhance meniscal healing with addition of fibrin clots or growth factors have shown some promise, consistent with the idea that the intrinsic healing potential of the meniscus might be improved by activation of endogenous meniscal stem cells (4). However, progress in this area has been limited by a lack of information about the origin of meniscal progenitors and the signaling pathways controlling their proliferation and differentiation.

Recently, mesenchymal stem cell (MSC) based therapies have been used to treat meniscal injuries as an alternative for surgical repair of meniscal lesions. Studies have shown that bone marrow derived MSCs, adipose derived MSCs and synovium derived MSCs can be used to enhance healing of meniscal defects in animal models (5-10). Although using MSCs has shown promise, tissue specific stem cells that reside in the meniscus are likely to have distinct characteristics that enable better repair of the meniscus, so that the tissue returns to the fully functional capacity required to prevent

joint degeneration (11-13). These include an intrinsic homing capacity that may allow meniscus-derived stem cells to migrate to the injury site (11,14) and the ability to form both chondrogenic and fibrous tissues, a unique feature of this heterogenous structure (13,15).

A greater understanding of the basic biology of meniscus-derived stem cells will be necessary for their application in cell based repair and tissue engineering strategies. To this end, we isolated and characterized meniscal stem progenitor cells (MSPCs) from adult mouse meniscus. We chose to analyze murine cells because our data could then be used to design *in vivo* studies on the regulatory mechanisms underlying meniscal regeneration, as the mouse is an ideal system for genetic manipulation. Mouse MSPCs exhibit the general features of tissue-specific stem cells isolated from other musculoskeletal tissues, including clonogenicity, multi-potency and expression of several common cell surface markers. In addition, adult mouse MSPCs express meniscus signature genes first identified in embryonic mouse meniscus that may be important for meniscal formation (16). We also show that markers associated with MSPCs localize in distinct regions of the adult mouse meniscus hypothesized to harbor cells capable of responding to meniscal injury, further strengthening the idea that this tissue contains stem/progenitor cells that may be directed toward meniscal repair.

Materials and methods

Mice

All mouse studies were approved by the Harvard Medical School Institutional Animal Care and Use Committee. C57Bl/6 mice were obtained from Charles River labs. Mice were maintained in a virus and parasite-free barrier facility and exposed to a 12 hour (hr) light/dark cycle.

Meniscal explant cell isolation and culture

For meniscal explant cultures, hindlimbs were removed from 8 week (wk), 6 month (mo) and 1 year (y) old mice and placed in a petri dish containing sterile phosphate buffered saline (PBS) on ice. The surrounding muscle was trimmed away and the knee joint dissected out. The patellar tendon was cut and the femur and tibia separated. The menisci remain attached to the tibia and were removed from the articular surface of the bone using a scalpel and fine curved scissors. The medial and lateral menisci were then placed in sterile PBS on ice. In the tissue culture hood, forceps were used to place 1 pair of menisci into each well of a 6 well plate. One drop (50 µl) of growth media consisting of Minimum Essential Medium Alpha ascorbic acid free (α-MEM) (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS), 10 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin was added to cover each menisci. The plate was incubated for 2 hours at 37°C; 5% CO_2; 42% humidity to allow the tissue to adhere and then 1.5 ml of media was added to each well. After 3 days, an additional 1.5

ml of growth media was added and cells began to grow out of the explanted menisci 5-7 days later.

Colony forming and multi-differentiation potential assays

For colony forming efficiency assays, MSPCs from 8wk old mice at passage 1 (P1) were cultured at 1000-2000 cells per 25cm^2 flask in growth media for 12 days and stained with 0.05% methyl violet (Sigma, St. Louis, MO). Only colonies containing more than 50 cells were counted.

For multi-differentiation assays, MSPCs from 8wk old mice were plated at colony forming density in 25cm^2 flasks and cultured in growth medium for 12 days. To analyze osteogenic potential, cells were switched to osteogenic media that consisted of growth media supplemented with 10 mM β-glycerol phosphate, 50 μg/ml ascorbic acid (Sigma) for another 7 days. Colonies were then stained with 0.5% Alizarin Red S (Sigma). To test adipogenic potential, cells were switched to adipogenic media consisting of growth media supplemented with 100 nM d□□□□□th□□□□□, 5 μg/ml insulin, 50 μM indomethacin (Sigma) for 7 more days. Colonies were then stained with 0.3% Oil Red O (Sigma). To assess chondrogenic potential, cells were switched to chondrogenic media consisting of growth media supplemented with 50 μg/ml ascorbic acid, 100 nM d□□□□□th□□□□□, 40 μg/ml L-proline, 2mM sodium pyruvate and 1% insulin transferrin-selenious acid mix (ITS+) (Sigma) for another 7 days. Flasks were then stained with Alcian Blue pH 1.0 (Sigma).

Flow Cytometry (FACS) analysis

P1 MSPCs from 8 wk old mice were suspended at a density of 1 x10^6 cells in FACS buffer (PBS, 1% FBS) containing 1-5 μg/ml of primary or control antibodies. After incubation for 30 minutes at 4^{o}C, the cells were washed 3 times with FACS buffer and suspended in 500 μl FACS buffer for the analysis. The following Fluorescein isothiocyanate (FITC), allophycocyanin (APC) or phycoerythrin (PE) coupled antibodies were used: CD34 (#11-0341; eBiosciences, San Diego, CA), CD44 (#17-0441; eBiosciences, San Diego, CA), CD73 (#12-0731 eBiosciences, San Diego, CA), CD105 (#17-1051; eBiosciences, San Diego, CA), Sca1 (#561077; BD Pharmigen, San Jose, CA) and CD90.2 (#60115FI.2; STEMCELL Technologies, Vancouver, BC). Samples were evaluated using a BDLSRII flow cytometer (BD Biosciences, San Jose, CA) and the data analyzed using the Flowjo software program (FLOWJO, Ashland, OR).

RNA isolation and quantitative real time PCR (qRT-PCR) analysis

Total RNA was isolated from MSPCs (P1) from 8 wk old mice using the RNEasy Plus Universal Kit (Qiagen, Valencia, CA). Reverse transcription was performed using EcoDry Premix Kit (Clontech, Mountain View, CA). Quantitative PCR was performed using FastStart Universal SYBR Greeen Master Mix (Roche, Nutley, NJ) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies) and primers as outlined in supplemental Table 1. Values were normalized to *cyclophilin B* using the 2-ΔΔCt method (17).

Immunohistochemistry

For immunohistochemistry, decalcified sections of 8 wk old murine knee joints were deparaffinized, rehydrated and incubated in citrate buffer pH 6.0 at 65^{o}C for 1 hr or in hylauronidase (Sigma) at 37^{o}C for 10 mins for antigen retrieval. Sections were then blocked and incubated with primary antibody overnight at 4^{o}C. Immunohistochemical detection was performed using a Vectastain ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions and visualized using a DAB peroxidase substrate kit (Vector Labs, Burlingame, CA) followed by counterstaining with Hematoxylin QS (Vector Labs, Burlingame, CA). Primary antibodies used were biglycan (1:100, Abcam, Cambridge, MA), CD44 (1:1000, Abcam, Cambridge, MA), IGF-1 (1:250 Abcam, Cambridge,MA) and lysyl oxidase (1:250, ThermoFisher, Waltham, MA).

Statistical analysis

Results are presented as the mean plus and minus standard deviation (SD). Data are based on triplicate reactions of at least three biological samples.

Results

Mouse meniscal explant culture

Based on explant culture procedures for human meniscus and cartilage (11,18), a protocol was developed for isolating meniscal progenitor cells from adult mouse meniscus. Using sterile technique, the knee joint was dissected from hindlimbs of 8 wk, 6 mo or 1 y old mice. The patellar tendon was cut away and the femur and tibia separated. The menisci remained attached to the tibia and were subsequently removed from the articular surface of the bone. The medial and lateral menisci were then placed in 6 well dishes with small amount of growth media covering each meniscal pair. The plate was incubated for 2 hours at 37°C with 5% humidified CO_2 to allow the tissue to adhere. More media was added to each well and the explants were fed every 3 days. After 5-7 days, cells began to grow out of the explanted menisci (Figure 1A). In culture, these meniscus-derived cells grew clonally and exhibited a spindle-shaped morphology (Figure 1B) typical of mesenchymal stem cells and similar to migratory meniscal cells from the human meniscus (11). The cells grew equally well out of both the lateral and medial menisci of mice of all ages tested, were successfully passaged after 14 days and grew well in monolayer. Subsequent experiments were all performed using meniscal explants from 8 wk old mice and the meniscus derived cells were designated MSPC (Meniscal Stem Progenitor Cells).

Mouse Meniscal Stem Progenitor Cells (MSPCs) exhibit stem cell like characteristics

To begin to characterize the stem cell potential of MSPCs, we examined their clonogenicity. Single cell suspensions were generated and cultured from 8 wk old mouse meniscal explants. A portion of these meniscal cells attached onto the plastic surface of the flask and colonies were visualized using methyl violet staining after 12 days (Figure 1C). A small population (2-3%) of the meniscus derived cells formed adherent colonies that varied in size, cell density and morphology, reflecting differences in cell proliferation rates and the inherent heterogeneous nature of meniscal tissue (Figure 1D-F).

Next, we examined whether MSPCs had the capacity to differentiate into various cell lineages (osteoblast, adipocyte, chondrocyte), a fundamental characteristic of stem cells. To test the osteogenic potential of this cell population, MSPC colonies were differentiated in osteogenic medium. After 7 days, Alizarin Red positive mineralized calcium deposits were detected (Figure 1G). After induction in adipogenic medium for 7 days, MSPC colonies showed accumulation of lipid droplets visualized by positive oil red O staining (Figure 1H). When differentiated in chondrogenic medium for 7 days, MSPC colonies stained positive for Alcian Blue indicating synthesis of proteoglycans by chondrocytes (Figure 1I). These results suggest that MSPCs can form colonies and are multipotential, two key features of mesenchymal stem cells.

FACS analysis

To confirm whether the MSPCs possess the established properties of stem cells, they were subjected to fluorescence-activated cell sorting (FACS) analysis for surface markers associated with stem cells (Figure 2A). We found that MSPCs (P1) were over 97% positive for CD44, a mesenchymal stromal cell marker and over 87% positive for

stem cell antigen-1 (Sca-1). In addition, cells were also positive for CD90.2 (54%) and CD73 (35.4%) and expressed low levels of CD105 (2.2%). MSPCs were negative for CD34 verifying the lack of contaminating hematopoietic cells. This flow cytometry profile is similar to that of human migratory meniscus progenitor cells (11) and suggests that mouse MSPCs have characteristics of mesenchymal stem cells.

Gene expression profile of MSPCs

To further characterize the MSPCs, we examined the expression profile of molecular markers of mature meniscus, cartilage, tendon and bone, and also genes we have shown previously to be associated with formation of the embryonic meniscus (16). RNA was isolated from MSPCs expanded to passage 1(P1) from 8 wk old mouse meniscal explants. qPCR analysis revealed that MSPCs exhibited high relative expression of *biglycan*, a small leucine rich proteoglycan found in the meniscus, and *collagen type I*, the major collagen of the outer fibrous region of the meniscus and intermediate levels of *decorin*, the other meniscal small structural proteoglycan and *Sox9,* a chondrogenic transcription factor (Figure 2B). In contrast, MSPCs had lower relative expression of the major adult meniscal ECM components *collagen type 2* and *aggrecan* and did not express significant amounts of tendon or bone markers (Figure 2B). Intriguingly, MSPCs expressed robust levels of the meniscus signature gene *lysyl oxidase* (*Lox*), an enzyme responsible for collagen cross-links in skeletal and connective tissue, as well as *Igf-1*, the major signaling pathway enriched in the developing meniscus (16) (Figure 2C). This pattern of gene expression in MSPCs reflects the heterogenous fibro-cartilaginous composition of the adult meniscus.

Localization of MSPCs

Finally, to verify the data obtained from our FACs and qPCR analyses with MSPC behavior *in vivo*, we examined the spatial localization of a select group of MSPC expressed factors using immunohistochemistry. CD44, biglycan, Lox and IGF-1 were all detected in the outer periphery of the meniscus in the fibro-chondrocytes of the superficial zone in knees of 8 wk old mice (Figure 3A-F). This superficial zone is thought to contain endogenous progenitor cells with regenerative capabilities (19). In addition, positive staining was seen for CD44, biglycan, Lox and IGF-1 in the fibroblast like cells of the outer vascular zone. This region is rich in collagen type I and has a higher capacity for healing and repair (15). These data confirm our *in vitro* studies and suggest that in the mouse, endogenous progenitor cells may reside in the superficial and outer region of the meniscus *in vivo*.

Discussion

A detailed understanding of the molecular and cellular events that underlie tissue repair is essential for addressing pathologies associated with injury, disease and aging in the musculoskeletal system. Little information exists about resident stem cell populations in the mouse meniscus, a model increasingly used to study joint diseases including OA. Our previous work, focused on the developmental biology of mouse meniscus, identifying key events mediating morphogenesis and the signaling pathways that direct this process (16,20). Here we report the isolation and characterization of resident meniscus stem/progenitor cells (MSPC) derived from adult mouse meniscus explant cultures. MSPCs demonstrate general properties of other stem cells including clonogenicity and multi-potentiality, express cell surface antigens associated with mesenchymal stem cells, and display elevated levels of meniscal marker genes. In addition, we observe a correlation between sites within the meniscus that are thought to harbor endogenous repair cells and the localization of proteins associated with MSPCs. We believe that identification of MSPCs provides a powerful tool for enhancing cell-based strategies focused on meniscal regeneration as there is increasing evidence that tissue-resident stem cells are critical for organ homeostasis and effective wound healing (21). Although MSCs and synovium-derived stem cells have been tested in meniscal injury models (5-10), endogenous stem/progenitor cells may be best suited for repair of the meniscus, a tissue with distinct composition, architecture and function in the knee joint.

In previous studies, meniscus-derived stem cells were identified and isolated from rabbit and human following tissue digestion (12,13,22). Unlike those investigations, the MSPCs described in our studies migrated out of whole explanted mouse menisci. Since cell motility is now recognized as a vital property of stem and progenitor cells (21), our MSPCs may represent a more promising cell population for meniscal repair. The MSPCs we identified express progenitor and stem cell markers that localize to the outer vascular zone and superficial zone of the adult meniscus, two areas thought to contain cells for repair (15,19). As these anatomical sites are distinct in character, the presence of MSPCs at both locations suggests the possibility of multiple stem cell niches within the meniscus. It is also unclear at this point if our mouse MSPCs are a mixed population of stem cells that migrated out of several sites within the meniscus or a single population of MSPCs that reside at multiple sites where they respond to specific niche cues. Lineage tracing studies to track cell behavior using transgenic mouse models will be useful in answering this question.

Our *in vitro* analyses of mouse MSPC revealed elevated expression levels of *Lox* and *Igf-1*, genes previously identified as highly expressed in embryonic meniscus (16). In the musculoskeletal system, Lox functions to covalently cross-link collagen fibers, thus enhancing tissue mechanical integrity. Stimulating endogenous Lox expression improves the biomechanical properties of both native and engineered connective tissues, including meniscus (23), so that expression of Lox by MSPC may be one way of strengthening newly laid down repair tissue. IGF-1 is a critical mediator of both bone and cartilage tissues and has shown promise as a stimulator of cell proliferation and ECM production by meniscal fibrochondrocytes (24,25). In the context of repair, IGF-1 treatment of

engineered meniscal constructs correlates with improved mechanical, biochemical and surface lubrication properties (26,27). As tissue regeneration often recapitulates aspects of embryonic tissue morphogenesis, the expression of Lox and IGF-1 by MSPCs may be an identifying characteristic that defines a reparative cell population resident in the adult mouse meniscus.

Our current findings support the notion that a greater understanding of the basic biology of meniscal stem/progenitor cells is needed to better inform cell based treatments for meniscal pathologies. Analysis of meniscus derived-stem cells in an animal model such as the mouse will allow for detailed studies of their behavior during injury and repair and of the regulatory pathways that guide these processes, critical steps in identifying therapeutic targets for the regeneration of diseased or injured meniscal tissue.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Figure Legends

Figure 1. Isolation and analysis of stem cell features of MSPCs. (A) Phase contrast microscopy of cells growing out of lateral meniscus of 8wk old mouse after 5 days in culture. (B) Higher power view showing the spindle shaped morphology of these cells. Colony formation analysis of MSPCs. (C) Colonies formed from 2000 MSPCs after 12 days in culture stained with methyl violet. (D-E) Morphology of sample colonies formed by MSPCs. (F) Colony forming efficiency of MSPCs. The results are shown as means ± SD of 3 flasks at each cell density. The multidifferentiation potential of MSPCs. (G) Alizarin Red S staining shows osteogenic differentiation of MSPCs. (H) Oil Red O staining shows adipogenic differentiation of MSPCs. (I) Alcian Blue staining shows chondrogenic differentiation of MSPCs.

Figure 2. Characterization of MSPCs. (A) Flow cytometry analysis of the expression of cell surface markers related to stem cells. MSPCs were 97.3% positive for CD44, 87.8% positive for Sca-1, 54% positive for CD90, 35.4% positive for CD73, 2.2% positive for CD105 and 0.1% positive for CD34. (B) qRT-PCR analysis for markers of skeletal and connective tissue in MSPCs. (C) qRT-PCR analysis of genes associated with embryonic meniscal formation in MSPCs. Data represent three biological replicates with *cyclophilin B* used for normalization. Error bars are means ± SD.

Figure 3. Localization of proteins associated with MSPCs in mouse knee. (A) Safranin O staining of 8 wk old mouse knee (4X magnification). Scale bar = 200μm. F, femur; T, tibia; A, anterior meniscal horn; P, posterior meniscal horn. (B) High power view of posterior meniscal horn (10X magnification). Scale bar = 100μm . Arrowheads point to the superficial zone. IZ, inner zone of meniscus; OZ, outer zone of meniscus. Immunohistochemistry for (C) CD44 (D) Biglycan (BGN) (E) Lysyl oxidase (Lox) and (F) IGF-1 proteins showed positive staining of cells in the superficial and outer zone of posterior horn of the 8 wk meniscus. Scale bar = 100μm.

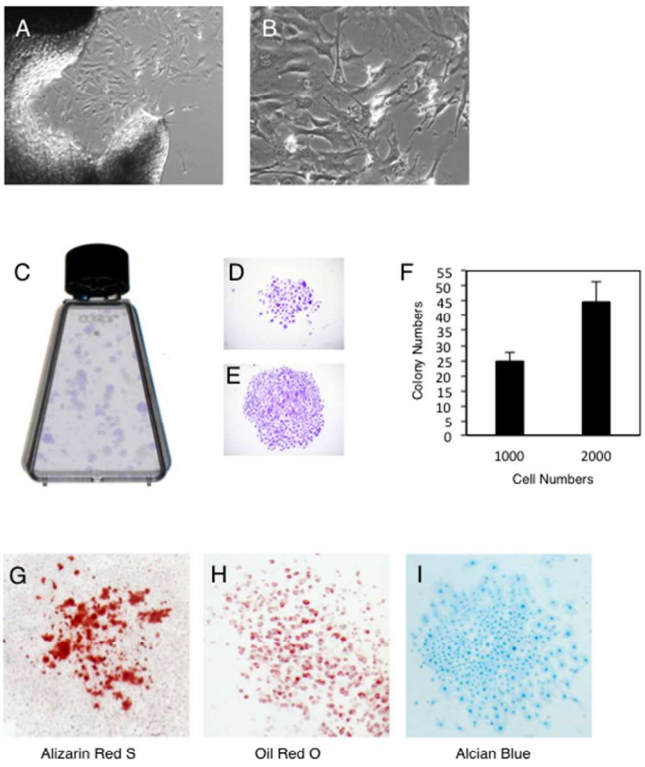

Figure 1

Figure 1

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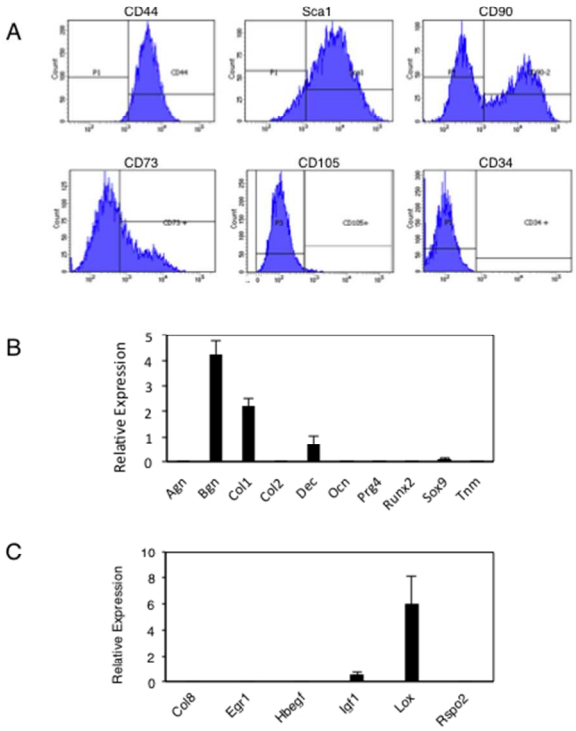

Figure 2

Figure 2

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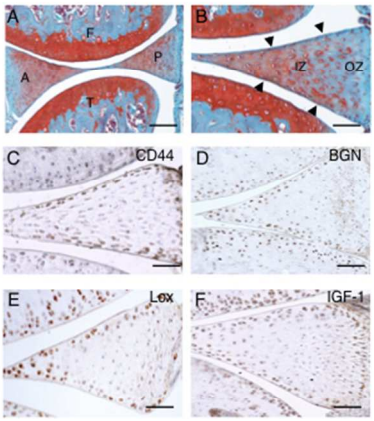

Figure 3

Figure 3

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Gene	Primer Pair (5'-3')
Cyclophilin B	TTCTTCATAACCACAGTCAAGACC ACCTTCCGTACCACATCCAT
Aggrecan	CCAGCCTACACCCCAGTG GAGGGTGGGAAGCCATGT
Biglycan	GAATGGTACCCACTTTGTACTGG GGAGCCTGCACCACAATAC
Col1a1	CATGTTCAGCTTTGTGGACCT GCAGCTGACTTCAGGGATGT
Col2a1	CGGTGGTACGGTGTCAGG TTATACCTCTGCCCATTCTGC
Col8a1	GGCCAGCCAAGCCTAAAT TGATGAACAGTATTCCCAGCA
Decorin	GAGGGAACTCCACTTGGACA TTGTTGTTGTGAAGGTAGACGAC
Egr1	CCTATGAGCACCTGACCACA TCGTTTGGCTGGGATAACTC
Hbegf	TCTTGTCATCGTGGGACTTCT CACGCCCAACTTCACTTTCT
Igf-1	GACCGAGGGGCTTTTACTTC CATCCACAATGCCTGTCTGA
Lox	CTCCTGGGAGTGGCACAG CTTGCTTTGTGGCCTTCAG

Osteocalcin	AGACTCCGGCGCTACCTT CTCGTCACAAGCCAGGGTTAAG
Prg4	GGCAAGTGCTGTGCAGATTA AGGCGGAGGTGCAGTCTT
Rspo2	CGCAGAGGTTGATAATTCACT TCCCTCCACGGTCCACTG
Runx2	CCACAAGGACAGAGTCAGATTACA TGGCTCAGATAGGAGGGGTA
Sox9	CAGCAAGACTCTGGGCAAG TCCACGAAGGGTCTCTTCTC
Tenomodulin	TGTACTGGATCAATCCCACTCTAA TTTTCACTGGTAGGAAAGTGAAGA